JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2007, 58, Suppl 1, 53–64
www.jpp.krakow.pl

K. ZWIRSKA-KORCZALA[1], M. ADAMCZYK-SOWA[1], P. SOWA[1], K. PILC[1], R. SUCHANEK[1], K. PIERZCHALA[2], G. NAMYSLOWSKI[3], M. MISIOLEK[3], K. SODOWSKI[4], I. KATO[5], A. KUWAHARA[6], R. ZABIELSKI[7]

ROLE OF LEPTIN, GHRELIN, ANGIOTENSIN II AND OREXINS IN 3T3 L1 PREADIPOCYTE CELLS PROLIFERATION AND OXIDATIVE METABOLISM

[1]Department of Physiology, Zabrze, Poland; [2]Department of Neurology, Zabrze, Poland; [3]Department of ENT, Zabrze, Poland; [4]Department of Obstetric and Gynecology, Ruda Slaska, Medical University of Silesia, Katowice, Poland; [5]Yanaihara Institute, Shizuoka, Japan; [6]Laboratory of Physiology, Institute of Environmental Sciences, University of Shizuoka, Shizuoka, Japan; [7]Department of Physiological Sciences, Warsaw Agricultural University, Warsaw, Poland

There is now growing evidence that the reactive oxygen species have an influence on proliferation and antioxidative status of various cell types. The aim of the study was to investigate the effects of different concentrations of leptin, ghrelin, angiotensin II and orexins on proliferation, culture medium malondialdehyde (MDA) levels and antioxidative enzymes activities: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in 3T3 L1 preadipocytes cell culture. Cell proliferation was measured using [^{3}H]tymidine incorporation. In 3T3-L1 cells leptin caused a significant reduction in proliferation (by 36%) compared to control. Ghrelin increased preadipocyte proliferation, and the effect was stronger in higher dose (by 39%), while proproliferatory effect of angiotensin II was stronger in lower doses (by 47%). All used doses of orexin A significantly increased 3T3 L1 cell proliferation (from 21% to 160%), while orexin B caused a marked reduction (from 35% to 70%) of this proliferation. The effects of both orexins were dose-dependent. Leptin and ghrelin increased activity of SOD, CAT, GSH-Px and decreased level of MDA. Angiotensin II treatment stimulated only SOD and CAT activities. Influence of orexins was different on various enzymes. Orexin A increased MDA levels, while orexin B caused a marked decrease in MDA levels. Our results strongly suggest the effects of appetite affecting hormones such as leptin and ghrelin on proliferation and antioxidative enzyme activities of preadipocyte cell lines. Orexin A was found to be the most efficient proliferative-signalling hormone, while orexin B revealed the most significant inhibitory effect on preadipocytes proliferation.

Key words: *leptin, ghrelin, angiotensin II, orexin A, orexin B, preadipocytes, proliferation, antioxidative enzymes, MDA*

INTRODUCTION

The regulation of body weight concerns the control of adipose tissue. Many studies have shown that adipose tissue is not only the passive energy store, but also an active endocrine organ, producing biologically active substances like adipocytokines, leptin, adiponectin, resistin and angiotensin (1).

Leptin (Ob protein), a protein product of the *ob* gene is produced and secreted mainly by adipose tissue cells and gastrointestinal tract. The presence of leptin and expression of its receptors (Ob-R) was detected in the central nervous system (CNS) and peripheral tissues. Leptin promotes body weight loss by acting on the brain to decrease food intake and to increase sympathetic nervous system activity (2). Moreover, leptin has been proposed to participate in acute phase response to inflammation. Endogenous and exogenous leptin exerts a potent gastroprotective activity by increasing gastric blood flow, local generation of nitric oxide and stimulation of transforming growth factor (TGF-α) production (3). The antioxidative enzymes (catalase and glutathione peroxidase) activities were found to be lower in the ob/ob mice and leptin treatment normalized these alterations (4). Furthermore, leptin reduced levels of malondialdehyde (MDA) in renal ischemia-reperfusion (I/R) damages in rats (5). Harmelen *et al.* demonstrated that during the preadipocyte differentiation leptin secretion was significantly increased (6).

Ghrelin, a 28-amino acid peptide, is an endogenous ligand for the growth hormone secretagogue (GHS) receptor. Two major molecular forms of ghrelin can be found in the stomach and plasma: acylated and desacylated ghrelin (7). There is widespread tissue expression of the ghrelingene. The highest level of ghrelin was found in the fundus of the stomach, but in lower levels it was also found in the small intestine, pancreatic islet cells, gallbladder, liver, spleen and even in the immune cells (8). The most important functions of ghrelin are appetite stimulation (by activation of neuropeptide Y and agouti-related protein release), positive energy balance, gastric motility and gastric acid secretion control, modulation of pancreatic exocrine and endocrine functions, modulation of the proliferation of neoplastic cells, as well as the immune system control (9). Ghrelin exhibits a strong gastroprotective role, at least in part due to its anti-inflammatory actions (10).

Orexin A and orexin B (also named hypocretins) are new hypothalamic peptides that stimulate food intake. Orexins act *via* two subtypes of G-protein coupled receptors OX1-R and OX2-R (11). In addition to the hypothalamus, orexins have been also found in the mucosa and neural plexuses of gastrointestinal tract (12) and pancreatic α- and β-cells (13). In 2006, Digby and collages demonstrated for the first time the presence of orexin receptors in human adipose tissue cells, suggesting a role of orexins in adipose tissue metabolism and adipogenesis (14). The gut may be controlled by orexins released from local neurons as well as secreted as hormones from endocrine cells. Ehrstrom *et al*.

suggested that there might exist a gut-adipocyte interaction in which high plasma orexin A concentration may stimulate food intake by decreasing plasma leptin level and thus interacting with leptin sensitive central mechanisms (15).

Both angiotensin II receptors (AT1 and AT2) expression can be found in 3T3-L1 preadipocytes. In human adipose tissue, both visceral and subcutaneous, a complete functional renin–angiotensin system is present (renin, angiotensin II, AT1 and AT2 receptors and angiotensin converting enzyme-ACE) (16 - 18). The activation of angiotensin II results in stimulation of prostacyclin production and release, which acts as a signal in the differentiation of preadipocytes to adipocytes (2).

It is known that a moderate increase of reactive oxygen species (ROS) can stimulate cell growth and proliferation. Potential harmful effects of ROS are eliminated by stimulation of antioxidative enzymes (19). The aim of the study was to investigate the effect of high and low concentrations of leptin, ghrelin, angiotensin II and orexins on proliferation of 3T3 L1 preadipocytes. Furthermore MDA level and antioxidative enzyme activities: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) in the cell culture medium were measured.

MATERIAL AND METHODS

Cell culture

3T3-L1 cells are preadipocytes obtained by Green and Kehinde from murine 3T3 fibroblasts by cloning clusters of cells filled with fat droplets. 3T3-L1 cells were purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). Preadipocytes were plateaued at a density of 5 x 10^5 per 10 cm^2 dishes and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin under an atmosphere of 95% air and 5% CO_2 at 37°C.

Experimental protocol

Cell cultures with or without leptin (10^{-11}M, 10^{-9}M and 10^{-6}M), ghrelin (10^{-11}M and 10^{-8}M), angiotensin II (2.5x10^{-9}M, 5x10^{-6}M and 10^{-4}M), orexin A (5x10^{-11}M, 10^{-10}M and 10^{-9}M) and orexin B (5x10^{-11}M, 10^{-10}M and 10^{-9}M) were incubated for 24 h. The investigated substances were applied separately. Incubation media were not changed during this time. After incubation period, media were removed, centrifuged and freezed until laboratory measurements. Cells were then trypsinized and used for proliferation assay. The effect on cell proliferation was measured using [^{3}H]thymidine incorporation method. Cells were exposed to 0.5 μCi [^{3}H]thymidine per well for 2 h (20).

Enzymatic assays

SOD (EC 1.15.1.1) activities were estimated according to Oyanagui and expressed in nitrite units/ml (NU/mL) (21). GSH-Px (EC 1.11.1.19), activities were measured according to Paglia and Valentine using enzymatic conjunction with glutathione reductase (μmol $NADPH_2$/mL medium) (22). CAT (EC 1.11.1.6), activities were measured according to kinetic method of Aebi and

expressed in IU/ml of medium (23). MDA concentrations were determined according to the colorimetric method by Ohkawa *et al*. using reaction with thiobarbituric acid (24).

Drugs

The following agents were used: trypsin, Penicillin-Streptomycin Mixture, Dulbecco's Modified Eagle's Medium and Fetal Bovine Serum (BioWhittaker, Verviers, Belgium), leptin, orexin A and orexin B, ghrelin, angiotensin II (Sigma-Aldrich, St. Louis, Missouri, USA) and [^{3}H]thymidyne (Amersham Biosciences, Buckinghamshire England).

Statistics

All results were calculated to a mean ± SEM and presented as percent of control. Student's *t*-test was used for comparing results. Differences with a $p<0,05$ were regarded as significant.

RESULTS

Cell proliferation

As shown in *Fig. 1*, leptin in all used concentrations (10^{-11}M, 10^{-9}M, 10^{-6}M) caused a marked decrease in cell proliferation after 24 h incubation time (for about 18%, 22% and 36%, respectively, $p<0.05$). Treatment with ghrelin (10^{-11}M and 10^{-8}M) or angiotensin II ($2.5x10^{-9}$M and $5x10^{-6}$M) resulted in a significant increase in cell proliferation for about 18% and 39% or 47% and 30%, respectively. Furthermore orexin A ($5x10^{-11}$M, 10^{-10}M and 10^{-9}M), caused a

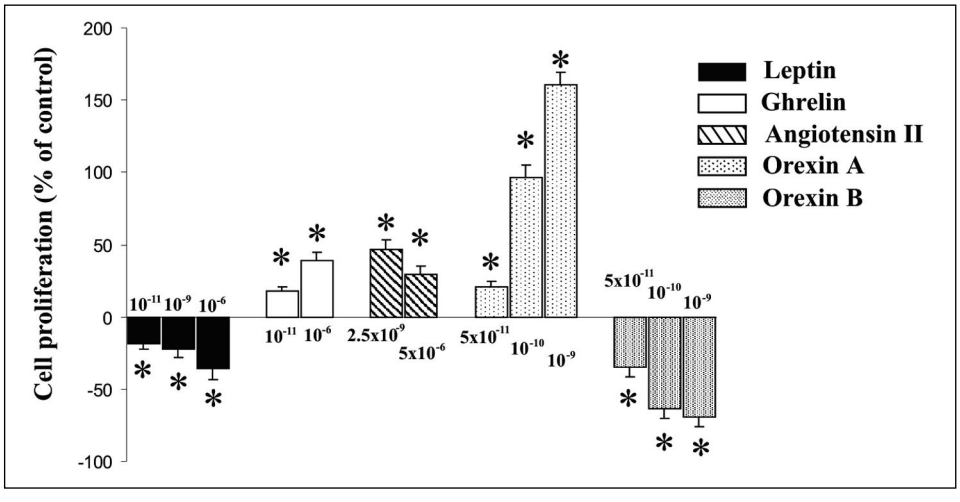

Fig. 1. Influence of leptin (10^{-11}M, 10^{-9}M, 10^{-6}M), ghrelin (10^{-11}M, 10^{-6}M), angiotensin II ($2.5x10^{-9}$M, $5x10^{-6}$M), orexin A ($5x10^{-11}$M, 10^{-10}M, 10^{-9}M) or orexin B ($5x10^{-11}$M, 10^{-10}M, 10^{-9}M) treatment on 3T3-L1 cell proliferation counted as [^{3}H]thymidine uptake. Data was presented as percent of control ± SEM.

significant increase in cell proliferation in dose-dependent manner (about 21%, 96% and 160%, respectively). Contrary incubation with orexin B ($5x10^{-11}$M, 10^{-10}M and 10^{-9}M) caused a marked dose-dependent decrease in cell proliferation (about 35%, 63% and 70%).

Antioxidative enzymes activity

Leptin treatment resulted in an increase in SOD and GSH-Px activities independently on the dose used, while CAT activity was increased in dose-dependent manner (*Fig. 2*). Ghrelin caused a dose-dependent increase in SOD activity, while CAT and GSH-Px activities were also increased but in reversed manner to the dose used (*Fig. 3*). Incubation with angiotensin II resulted in an increase in SOD and CAT activities (*Fig. 4*), but the influence on GSH-Px is negligible (data not shown). Influence of orexin A on enzyme activities revealed a dose-dependent decrease in CAT activity and a dose-dependent increase in GSH-Px activity, while there was no statistical changes in SOD activity (*Fig. 5*). Orexin B caused about 20% decrease in SOD activity, increased about 20% CAT and 40% GSH-Px activities, similarly in all used concentrations (*Fig. 6*).

MDA concentrations

Treatment with leptin resulted in about 5-10% decrease in MDA levels, in dose-dependent manner (*Fig. 2*). Ghrelin caused about 20% decrease in both used

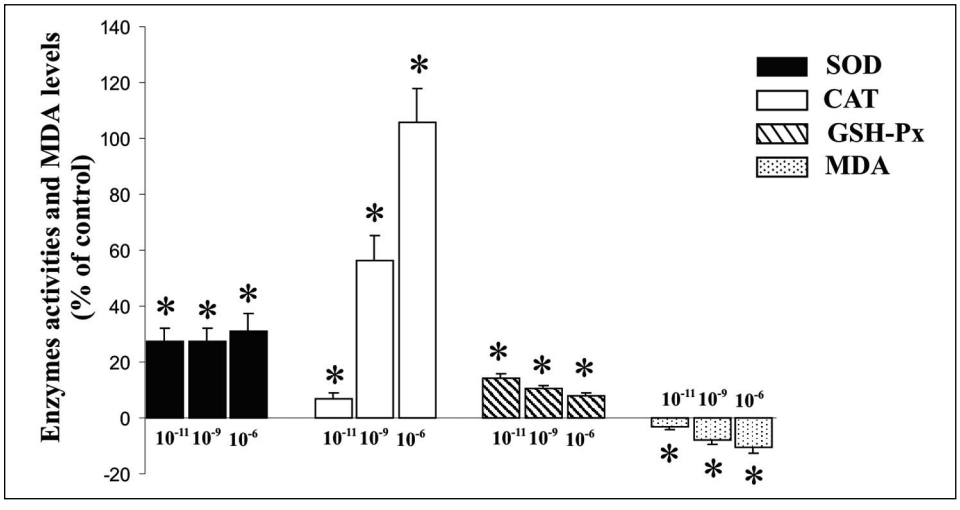

Fig. 2. Influence of leptin (10^{-11}M, 10^{-9}M, 10^{-6}M) on antioxidative enzymes activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in 3T3-L1 cell medium. Data was presented as percent of control ± SEM.

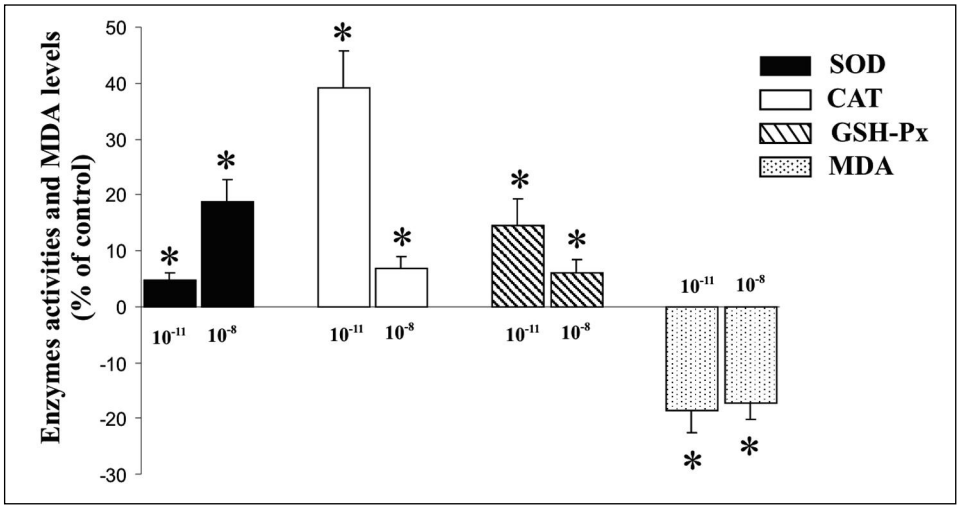

Fig. 3. Influence of ghrelin (10^{-11}M, 10^{-6}M) on antioxidative enzymes activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in 3T3-L1 cell medium. Data was presented as percent of control ± SEM.

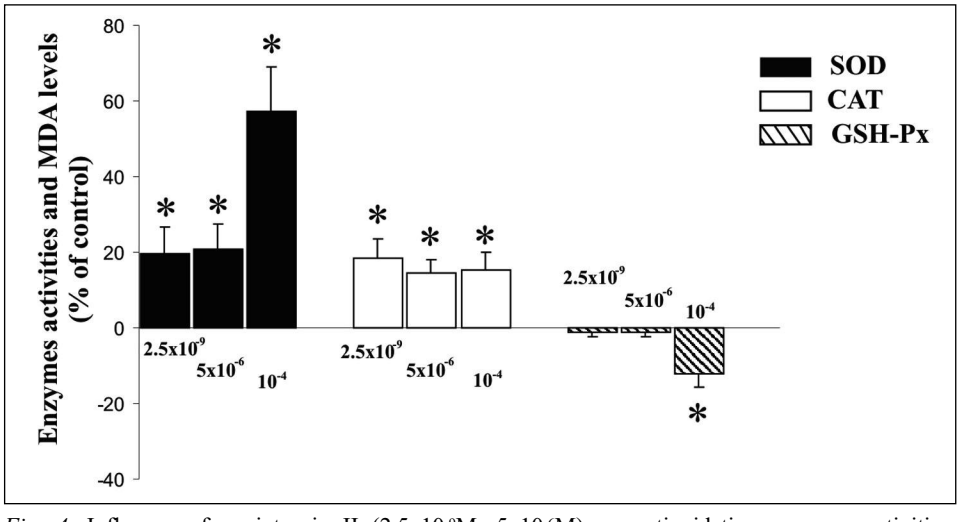

Fig. 4. Influence of angiotensin II ($2.5x10^{-9}$M, $5x10^{-6}$M) on antioxidative enzymes activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in 3T3-L1 cell medium. Data was presented as percent of control ± SEM.

concentrations (*Fig. 3*), while angiotensin II significantly decreased MDA levels only in the higher dose (in concentration 10^{-4}M for about 12%) (*Fig. 4*). Orexin A increased, while orexin B decreased MDA levels, and those actions were the most significant in the higher concentrations (*Figs 5* and *6*).

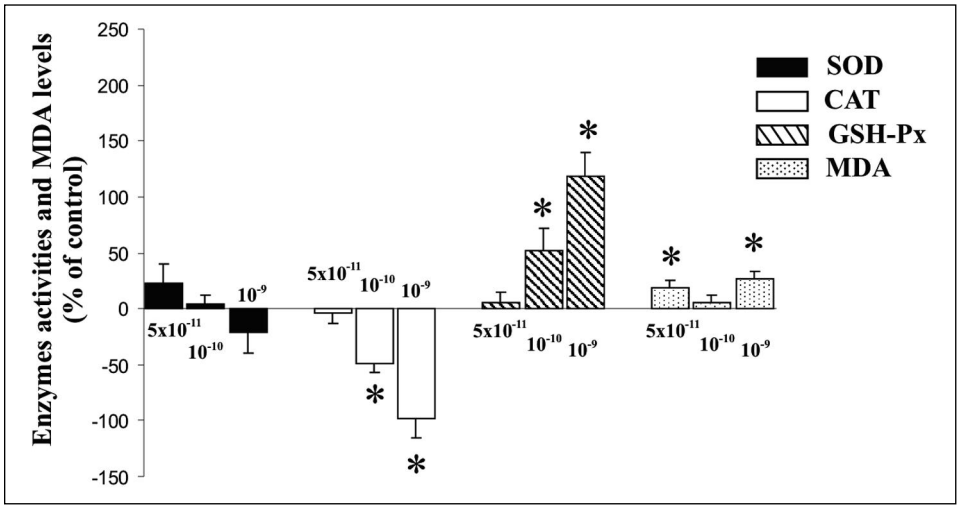

Fig. 5. Influence of orexin A (5x10^{-11}M, 10^{-10}M, 10^{-9}M) on antioxidative enzymes activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in 3T3-L1 cell medium. Data was presented as percent of control ± SEM.

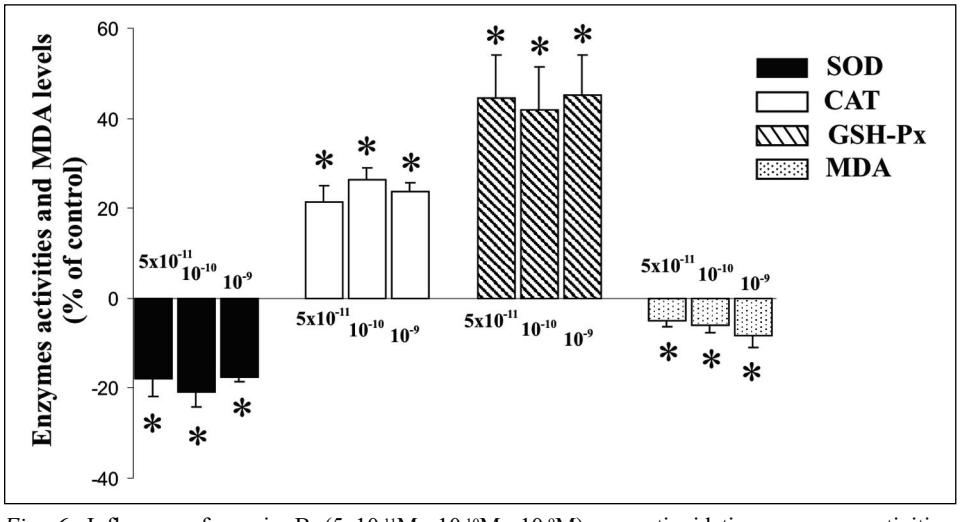

Fig. 6. Influence of orexin B (5x10^{-11}M, 10^{-10}M, 10^{-9}M) on antioxidative enzymes activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) levels in 3T3-L1 cell medium. Data was presented as percent of control ± SEM.

DISCUSSION

The present study demonstrates that leptin treatment causes a marked decrease in preadipocyte proliferation. Similarly, in the work of Schulz *et al*., leptin (50

ng/mL) decreased cell proliferation in mouse trophoblast cells (25). Harmelen *et al.* demonstrated that during the differentiation of preadipocytes leptin secretion significantly increased. Leptin secretion was about two- to eightfold higher in subcutaneous than in omental cells (6). Our study demonstrates that ghrelin at concentration of 10^{-11}M and 10^{-8}M stimulates proliferation of 3T3-L1 cells. Similar data were presented by other authors. Kim *et al*. showed that ghrelin at concentrations from 10^{-13}M to 10^{-7}M increased adipocyte cell number in 3T3-L1 culture cells, with the strongest effect observed in 10^{-11}M concentration (26). Zhang *et al.* have shown that in 3T3-L1 cells exogenous ghrelin treatment (in concentration 1μM) stimulated preadipocyte proliferation (27). Stimulatory effect of ghrelin in rat osteoblasts was observed at low concentrations (from 10^{-11}M to 10^{-9} M) but not in higher concentrations (from 10^{-9} to 10^{-8} M) (28). Similarly, dose-dependent effect of ghrelin, reaching maximal stimulation at concentration of 1nM, in human osteoblasts was observed (29).

Our results suggest a stimulatory effect of angiotensin II in doses of $2.5x10^{-9}$M and $5x10^{-6}$M, on preadipocytes supporting previously described data. In human preadipocytes 1 μM of angiotensin II reduced the relative proportion of preadipocytes in G1- and increased the number of cells in the S- and G2-M phases. The 6 h treatment of preadipocytes with angiotensin II was associated with a significant dose-dependent increase in the expression of cyclin D1 mRNA that is necessary for proliferation (30). Angiotensin II *in vitro* stimulated proliferation of rat vascular smooth muscle cells (VSMCs) (31, 32), arterial smooth muscle cells (RASMC) (33) and aortic smooth muscle cells (SMC) (34). Angotensin II also significantly increased intracellular superoxide anion (O^2-) generation. Cell proliferation of VSMCs was associated with the generation of ROS because diphenylene iodonium (DPI), a nonspecific NADPH oxidase inhibitor, blocked the mitogenic effect induced by the increase in ROS production (31).

Our study showed that orexin A applied at concentrations of $5x10^{-11}$M, 10^{-10}M and 10^{-9}M increased 3T3-L1 cell proliferation in dose dependent manner and orexin B treatment at concentrations of $5x10^{-11}$M, 10^{-10}M and 10^{-9}M resulted in a decrease of cell proliferation. The results confirm earlier findings by Spinazzi *et al*. that orexin B at concentration of 10^{-8}M and 10^{-6}M decreased proliferogenic activity of cultured rat adrenocortical cells, and the effect was inhibited by OX2-R immuno-blocade, suggesting an antiproliferogenic activity of OX2-Rs. Orexin A enhanced proliferogenic activity, indicating that OX1-R activation mediates this effect and this effect was inversely correlated with the peptide concentration. The authors suggested that at concentration of 10^{-10}M orexin A exclusively activated OX1-R, while at concentration of 10^{-6}M activated not only OX1-R but also OX2-R (35). Dose dependent differences in orexin A effects between our results and previous study may be related to a lower peptide concentrations used in our experiment.

In the present study, leptin treatment caused a significant increase in SOD, CAT and GSH-Px activities. Chronic leptin treatment resulted in stimulation of

fatty acids oxidation, inhibition of glucose oxidation and lipogensis in porcine adipocytes (36). The antioxidative enzymes: CAT and GSH-Px activities were lower in the ob/ob mice and leptin treatment reversed these alterations. Moreover, leptin had no effect on CAT and GSH-Px activity in lean mice, but caused a significant increase in glutathione reductase activity in both lean and ob/ob mice (4). Leptin increased ROS production in bovine aortic endothelial cells in a dose-dependent manner and increased fatty acid oxidation (37).

Increased MDA and protein carbonyls (PCs) levels are markers of oxidative stress. Leptin reduced levels of MDA and renal tissue PCs contents in renal ischemia-reperfusion damages in rats (5). Similarly in our study leptin treatment resulted in MDA decrease in all used doses. Pro-inflammatory cytokines, TNF-α and IL-1β, decreased leptin secretion in human adipose tissue *in vitro*. In contrast, IL-6 and IL-8 had no effects on leptin secretion and expression (38).

In our work ghrelin, similarly to leptin, caused a significant increase in SOD, CAT and GSH-Px activities and reduced MDA levels. Iseri and colleagues have shown that ghrelin treatment significantly increased GSH-Px activities and reduced MDA levels in the alendronate-induced gastric tissue injury in rats. Also ghrelin decreased formation of ROS (39).

Treatment of angiotensin II in our study resulted in an increase in SOD and CAT activities. MDA levels were reduced only after incubation with the higher used dose (10^{-4}M). Angiotensin II enhanced both the activity and the expression of the NAD(P)H oxidase and increased ROS generation, mainly mediated *via* AT1 receptors *in vitro* (31, 40 - 42) and *in vivo* (43). Rueckschloss *et al.* showed a dose-depend increase in NAD(P)H oxidase expression after angiotensin II treatment in primary cultures of human endothelial cells (40). Seshiah *et al.* have revealed that angiotensin II stimulated NADPH oxidase activity in VSMCs, leading to superoxide (O_2^-) and H_2O_2 generation (41). Subcutaneous infusions of angiotensin II in rats decreased the mRNA expression for extracellular superoxide dismutase by 55% and increased renal expression of NADPH oxidase (44). In spontaneously hypertensive rats extracellular SOD activities were significantly reduced and NAD(P)H oxidase complex significantly increased after angiotensin II treatment (42). *Ex vivo* in skin fibroblasts from adolescents with diabetic angiopathy, irbesartan, a selective antagonist of AT1 receptor, increased the enzymatic activity and mRNA expression of antioxidative enzymes: CuZnSOD, MnSOD, CAT, GSH-Px and reduced lipid peroxidation and MDA levels (45).

Influence of orexins on antioxidant enzymes activities is still unknown. Results from our study reveal different effects of orexins on antioxidative status in 3T3-L1 preadipocytes. Interestingly, orexin A increased MDA levels and stimulated cell proliferation. On the other hand, orexin B decreased MDA levels and decreased cell proliferation. These findings are in keeping with previous data that a moderate increase of ROS is able to stimulate cell cycle progression and promote cell proliferation. However, it is unclear which ROS species is mainly

responsible for stimulating cell proliferation. Activities of antioxidative enzymes may play an important role in regulation of cell proliferation and adipose tissue development through their abilities to regulate the level of cellular ROS level, known to be involved in cell growth and proliferation.

In summary, orexin A was found to be the most efficient proliferative-signalling hormone, on the other hand, orexin B showed the most significant inhibitory effect on preadipocytes proliferation. We concluded that leptin, ghrelin, angiotensin II and orexins participate in white adipose tissue metabolism by influencing antioxidative status and proliferation of preadipocytes.

REFERENCES

1. Wiecek A, Kokot F, Chudek J, Adamczak M. The adipose tissue - a novel endocrine organ of interest to the nephrologist. *Nephrol Dial Transplant* 2002; 17(2): 191-195.
2. Trayhurn P, Beattie J. Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. *Proceedings Nutr Soc* 2001; 60: 329-339.
3. Warzecha Z, Dembinski A, Ceranowicz P, et al. Influence of leptin administration on the course of acute ischemic pancreatitis. *J Physiol Pharmacol* 2002; 53(4): 775-790.
4. Watson AM, Poloyac SM, Howard G, Blouin RA. Effect of leptin on cytochrome P-450, conjugation, and antioxidant enzymes in the ob/ob mouse. *Drug Metab Dispos* 1999; 27(6): 695-700.
5. Erkasap S, Erkasap N, Koken T, et al. Effect of leptin on renal ischemia-reperfusion damage in rats. *J Physiol Biochem* 2004; 60(2): 79-84.
6. van Harmelen V, Dicker A, Ryden M, et al. Increased lipolysis and decreased leptin production by human omental as compared with subcutaneous preadipocytes. *Diabetes* 2002; 51(7): 2029-2036.
7. Nanzer AM, Khalaf S, Mozid AM, et al. Ghrelin exerts a proliferative effect on a rat pituitary somatotroph cell line via the mitogen-activated protein kinase pathway. *Eur J Endocrinol* 2004; 151(2): 233-240.
8. Gnanapavan S, Kola B, Bustin SA, et al. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* 2002; 87: 2988-2991.
9. Lely van der AJ, Tschop M, Heiman ML, et al. Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev* 2004; 25: 426-457.
10. Konturek PC, Brzozowski T, Pajdo R, et al. Ghrelin – a new gastroprotective factor in gastric mucosa. *J Physiol Pharmacol* 2004; 55(2): 325-336.
11. Ammoun S, Holmqvist T, Shariatmaderi R, et al. Distinct recognition of OX1 and OX2 receptors by orexin peptides. *J Pharm Exp Ther* 2003; 305: 507-514.
12. Dalal MA, Schuld A, Haack M, et al. Normal plasma levels of orexin A (hypocretin-1) in narcoleptic patients. *Neurology* 2001; 56: 1749-1751.
13. Ouedraogo R, Naslund E, Kirchgessner AL. Glucose regulates the release of orexin-A from the endocrine pancreas. *Diabetes* 2003, 52: 111-117.
14. Digby JE, Chen J, Tang JY, Lehnert H, Matthews RN, Randeva HS. Orexin receptor expression in human adiposa tissue: effects of orexin A and orexin B. *J Endocrinol* 2006; 191(1): 129-136.
15. Ehrstrom M, Gustafsson T, Finn A, et al. Inhibitory effect of exogenous orexin A on gastric emptying, plasma leptin, and the distribution of orexin and orexin receptors in the gut and pancreas in man. *J Clin Endocrinol Metab* 2005; 90: 2370-2377.

16. Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM. Mature adipocytes inhibit in vitro differentiation of human preadipocytes via angiotensin type 1 receptors. *Diabetes* 2002; 51(6): 1699-1707.
17. Schling P, Loffler G. Effects of angiotensin II on adipose conversion and expression of genes of the renin-angiotensin system in human preadipocytes. *Hormon Metab Res* 2001; 33(4): 189-195.
18. Giacchetti G, Faloia E, Mariniello B, et al. Overexpression of the renin-angiotensin system in human visceral adipose tissue in normal and overweight subjects. *Am J Hyperten* 2002; 15(5): 381-388.
19. Hu Y, Rosen DG, Zhou Y, et al. Role in cell proliferation and response to oxidative stress. *J Biol Chem* 2005; 280(47): 39485-39492.
20. Bachmeier M, Loffler G. Influence of growth factors on growth and differentiation of 3T3-L1 preadipocytes in serum-free conditions. *Eur J Cell Biol* 1995; 68: 323-329.
21. Oyanagui Y. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. *Anal Biochem* 1984; 142: 290-296.
22. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967; 70: 158-169.
23. Aebi H. Catalase *in vitro*. *Methods Enzymol* 1984; 105: 121-126.
24. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-358.
25. Schulz LC, Widmaier EP. The effect of leptin on mouse trophoblast cell invasion. *Biol Reprod* 2004; 71(6): 1963-1967.
26. Kim MS, Yoon CY, Jang PG, et al. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. *Mol Endocrinol* 2004; 18(9): 2291-2301.
27. Zhang W, Zhao L, Lin TR, et al. Inhibition of adipogenesis by ghrelin. *Mol Biol Cell* 2004; 15(5): 2484-2491.
28. Maccarinelli G, Sibilia V, Torsello A, et al. Ghrelin regulates proliferation and differentiation of osteoblastic cells. *J Endocrinol* 2005; 184(1): 249-256.
29. Delhanty PJ, van der Eerden BC, van der Velde M, et al. Ghrelin and unacylated ghrelin stimulate human osteoblast growth via mitogen-activated protein kinase (MAPK)/phosphoinositide 3-kinase (PI3K) pathways in the absence of GHS-R1a. *J Endocrinol* 2006; 188(1): 37-47.
30. Crandall DL, Armellino DC, Busler DE, McHendry-Rinde B, Kral JG. Angiotensin II receptors in human preadipocytes: role in cell cycle regulation. *Endocrinology* 1999; 140: 154-158.
31. Li PG, Xu JW, Ikeda K, et al. Caffeic acid inhibits vascular smooth muscle cell proliferation induced by angiotensin II in stroke-prone spontaneously hypertensive rats. *Hypertens Res* 2005; 28(4): 369-377.
32. Nagata D, Takeda R, Sata M, et al. AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation. *Circulation* 2004; 110(4): 444-451.
33. Kyaw M, Yoshizumi M, Tsuchiya K, et al. Antioxidant effects of stereoisomers of N-acetylcysteine (NAC), L-NAC and D-NAC, on angiotensin II-stimulated MAP kinase activation and vascular smooth muscle cell proliferation. *J Pharmacol Sci* 2004; 95(4): 483-486.
34. Yang X, Zhu MJ, Sreejayan N, Ren J, Du M. Angiotensin II promotes smooth muscle cell proliferation and migration through release of heparin-binding epidermal growth factor and activation of EGF-receptor pathway. *Mol Cells* 2005; 20(2): 263-270.
35. Spinazzi R, Ziolkowska A, Neri G, et al. Orexins modulate the growth of cultured rat adrenocortical cells, acting through type 1 and type 2 receptors coupled to the MAPK p42/p44- and p38-dependent cascades. *Int J Molecular Med* 2005; 15: 847-852.

36. Ramsay TG. Porcine leptin alters insulin inhibition of lipolysis in porcine adipocytes in vitro. *J Anim Sci* 2001; 79(3): 653-657.
37. Yamagishi S, Edelstein D, Du X, Kaneda Y, Guzman M, Brownlee M. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem* 2001; 276(27): 25096-25100.
38. Bruun JM, Pedersen SB, Kristensen K, Richelsen B. Effects of pro-inflammatory cytokines and chemokines on leptin production in human adipose tissue in vitro. *Mol Cell Endocrinol* 2002; 190(1-2): 91-99.
39. Iseri SO, Sener G, Yuksel M, et al. Ghrelin against alendronate-induced gastric damage in rats. *J Endocrinol* 2005; 187(3): 399-406.
40. Rueckschloss U, Quinn MT, Holtz J, Morawietz H. Blockade in patients with coronary artery disease in human endothelial cells: protective effect of angiotensin II type 1 receptor dose-dependent regulation of NAD(P)H oxidase expression by angiotensin II. *Arterioscler Thromb Vasc Biol* 2002; 22:1845-1851.
41. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. *Circ Res* 2002; 91: 406-413.
42. Adler S, Huang H. Oxidant stress in kidneys of spontaneously hypertensive rats involves both oxidase overexpression and loss of extracellular superoxide dismutase. *Am J Physiol Renal Physiol* 2004; 287: F907-F913.
43. Lopez M, Senaris R, Gallego R, et al. Orexin receptors are expressed in the adrenal medulla of the rat. *Endocrinology* 1999; 140: 5991-5994.
44. Welch WJ, Blau J, Xie H, Chabrashvili T, Wilcox ChS. Angiotensin-induced defects in renal oxygenation: role of oxidative stress. *Am J Physiol Heart Circ Physiol* 2005; 288: H22-H28.
45. Chiarelli F, Di Marzio D, Santilli F, et al. Effects of irbesartan on intracellular antioxidant enzyme expression and activity in adolescents and young adults with early diabetic angiopathy. *Diabetes Care* 2005; 28(7): 1690-1697.

Received: January 25, 2007
Accepted: February 20, 2007

Author's address: Prof. Krystyna Zwirska-Korczala, Department of Physiology, Medical University of Silesia, 41-800 Zabrze, ul. Jordana 19, Poland. E-mail: kkorcz@slam.katowice.pl